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Construction of a Recombinant Viral Vector Containing Part of the Nucleocapsid Protein Gene of Newcastle Disease Virus (U)

BY

D. E. Bader

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**SUFFIELD
MEMORANDUM
NO. 1464**

CONSTRUCTION OF A RECOMBINANT VIRAL VECTOR CONTAINING PART OF THE NUCLEOCAPSID PROTEIN GENE OF NEWCASTLE DISEASE VIRUS

BY

Douglas E. Bader

SEPTEMBER 1995

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ABSTRACT

This report describes the procedures used to clone a 673 base pair gene fragment of the major nucleocapsid protein gene of Newcastle disease virus into a viral vector molecule for the purpose of maintaining a stable, long-term, renewable source of this target sequence for gene probe studies.

The gene fragment was prepared by reverse-transcription polymerase chain reaction of Newcastle disease virus RNA and was cloned into the viral DNA vector M13mp18 RF to produce a recombinant DNA molecule. The cloned fragment was shown to be present in the recombinant clones based on (i) clonal selection on indicator plates; (ii) restriction enzyme analysis; (iii) gene probe analysis and (iv) nested PCR amplification.

EXECUTIVE SUMMARY

Title Bader, D. E. "Construction of a Recombinant Viral Vector Containing Part of the Nucleocapsid Protein Gene of Newcastle Disease Virus". *DRES Suffield Memorandum No. 1464*, 1995.

Introduction The Canadian Forces have a basic requirement to be able to function within a biological warfare (BW) environment. Early warning detection and identification of biological agents is the first line of defence against an attack. A key technology that is being studied at Defence Research Establishment Suffield (DRES) for identification of biological agents is gene probe technology. Gene probes are pieces of genetic material that are selected to match unique target nucleic acid sequences of suspected biological agents through the process of complementary molecular recognition. Gene probes play a key role in identification of biological agents because of the capability to identify genetic material regardless of its source, thereby affording identification of conventional agents such as viruses, bacteria, rickettsia or fungi, as well as potential threats, such as harmless organisms containing cloned threat agent genes or micro-encapsulated infectious nucleic acid.

Gene probe identification of very small quantities of target nucleic acid is made possible by using molecular biological techniques which increase the number of copies of a target sequence (amplification). One of these techniques, called polymerase chain reaction or PCR, uses short DNA sequences called primers, that bind to the DNA target sequence and set the boundaries of the region of the DNA to be amplified (when RNA is the target for PCR amplification, the RNA must be converted to DNA prior to PCR using an enzyme called reverse transcriptase). The PCR process generates multiple copies of this DNA sequence with a defined size or length. Size information can be used as a means of identification, if the primers complement unique or agent-specific sites on the target sequence. Further evidence that the correct DNA fragment has been amplified, can be obtained by probing the amplified product with a gene probe that is designed to bind to a target sequence within the amplified fragment.

We have designed PCR primers and gene probes for Newcastle disease virus (NDV). This has been done for two reasons. First, NDV has been developed as a BW viral simulant for use in field experiments at DRES.

Second, many viruses that could be potential BW threats are RNA viruses and since the genomic nucleic acid component of NDV is RNA, NDV serves as a useful model for developing methodologies for the identification of RNA viruses. The PCR primers and gene probes thus far used, have been designed for the major nucleocapsid protein gene. This gene was selected as a target because it is part of the nucleocapsid structure unit of the virus which has been suggested to be a necessary requirement for negative-stranded RNA viruses. Because of its importance to the virus, it is likely that this structure is highly conserved among the negative-stranded RNA viruses and therefore this gene should be a good target with which to design negative-stranded RNA viral gene probes. Part of the major nucleocapsid gene sequence has been generated by RT-PCR and used in DRES studies. This report describes the process of cloning this gene sequence into a viral DNA vector molecule to construct a recombinant DNA molecule which is easily stored within and recovered from a bacterial host cell line, thereby allowing us the capability to maintain and recover this sequence in unlimited quantities for future studies.

Results

The nucleocapsid protein gene fragment was successfully cloned into the viral vector M13mp18 RF to produce a recombinant DNA molecule. The recombinant DNA was shown to contain the cloned fragment based on four lines of evidence. First, bacterial cells transformed with the recombinant DNA, formed clear plaques in the presence of indicator dyes while bacterial cells transformed with vector DNA, produced blue plaques. Second, recombinant DNA that was isolated from clear plaques and was treated with the same restriction enzymes that were used in the cloning process, generated a fragment of similar size to the cloned gene fragment. Third, a positive signal was generated for the recombinant DNA but not for the vector DNA, following hybridization with a gene probe designed to bind to the cloned gene fragment. Finally, PCR amplification of the recombinant DNA using a set of nested PCR primers (primers located within the original primer pair) generated an amplified product (amplicon) of the expected size following agarose gel electrophoresis. The nested amplicon was probed with the NDV gene probe and found to generate a positive signal.

*Significance
of Results*

The cloned nucleocapsid gene fragment will allow DRES to maintain an unlimited source of this sequence for future studies.

Future Goals

The recombinant DNA will be maintained inside *E. coli* host cells and isolated for future studies when required.

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**GLOSSARY OF TERMS
AND ABBREVIATIONS**

agarose gel electrophoresis: the procedure by which DNA fragments are separated based on size by molecular sieving through agarose in an electric field.

amplification: the process of making multiple copies of a DNA sequence.

amplicon: a DNA fragment of defined size generated by PCR amplification.

bp: an abbreviation for base pairs of DNA .

cDNA: single-stranded DNA complementary to an RNA, synthesized from it by *in vitro* reverse transcription.

cloning: the process of splicing a DNA fragment into another DNA molecule.

CIP: an abbreviation for calf intestinal alkaline phosphatase, an enzyme that catalyzes the removal of 5' phosphates from DNA and RNA.

dATP: 2'-deoxy-adenosine-5'-triphosphate.

dCTP: 2'-deoxy-cytidine-5'-triphosphate.

dGTP: 2'-deoxy-guanosine-5'-triphosphate.

dTTP: thymidine-5'-triphosphate.

dUTP: 2'-deoxy-uridine-5'-triphosphate.

dNTP: a generic abbreviation for deoxynucleotide triphosphates.

DNA: 2' deoxyribonucleic acid, the genetic coding sequence of cells.

DNase: short form for deoxyribonuclease, an enzyme that hydrolyzes or degrades DNA.

lac Z Gene: this gene is present in the cloning site of the M13mp18 RF vector DNA molecule and codes for β -galactosidase activity. When a DNA fragment is cloned into this site, the *lac Z* gene no longer codes for functional activity. This is used to select recombinant vector molecules containing cloned DNA fragments.

LB: an abbreviation for Luria-Bertani, a bacterial growth media containing yeast extract, tryptone and sodium chloride.

Ligation: the process of forming a phosphodiester bond to link two adjacent nucleotides separated by a nick in the same DNA strand (this process is opposite to that of restriction although it is not sequence specific).

LMP agarose: abbreviation for low melting point agarose, used in the purification of DNA following DNA fragment separation.

M13mp18 RF: A double-stranded viral vector DNA molecule that is designed for cloning genes or gene fragments.

NDV: abbreviation for Newcastle disease virus, a virus of the Paramyxoviridae virus family whose natural host is the chicken and whose genetic material is single-stranded RNA.

NDVNP673: the designated name for the 673 bp DNA fragment of Newcastle disease virus nucleocapsid protein gene that was generated by RT-PCR in this study.

NP: abbreviation for nucleocapsid protein.

PCR: abbreviation for polymerase chain reaction, a technique for making multiple copies of a size-specific DNA fragment.

Recombinant DNA: (in this paper) refers to the M13mp18 RF vector molecule containing the cloned 673 bp NDV NP gene fragment.

Restriction: the process by which the phosphodiester backbone is cut or nicked between two adjacent nucleotides of the DNA molecule with a restriction enzyme, usually at a sequence-specific location.

Restriction enzymes: enzymes that recognize sequence specific sites on the DNA molecule and nick the DNA.

RNA: ribonucleic acid.

RNase: short form for ribonuclease, an enzyme that hydrolyzes or degrades RNA.

RT: abbreviation for reverse transcription, the process by which RNA is converted to a single-stranded DNA copy.

RT-PCR: abbreviation for reverse transcription polymerase chain reaction, the process by which RNA is converted to multiple copies of a double-stranded DNA fragment of a defined size or length.

Southern Blot: the procedure by which denatured DNA is transferred from an agarose gel to a nylon filter where it is hybridized with a complementary DNA sequence (gene probe).

Tm: abbreviation for melting temperature, the midpoint of the temperature range over which double-stranded DNA is denatured to single-strands.

Vector DNA: (in this paper) refers to M13mp18 RF DNA which was used in this study for cloning.

X-gal: 5-bromo-4-chloro-3-indolyl- β -d-galactoside, a colorless substrate which is converted to blue-colored product in the presence of the enzyme β -galactosidase and IPTG.

INTRODUCTION

Genetic technologies play a key role in the strategies being developed at DRES for the identification of biological agents. Genetic technologies currently being studied at DRES include gene probe hybridization and polymerase chain reaction (PCR) amplification.

Gene probes are nucleic acid molecules that bind to complementary nucleic acid sequences (RNA or DNA) in a sequence specific manner, through the process of hybridization. Gene probes can be used for identifying organisms at almost all levels of the taxonomic classification level (family, order, genus, species and strain). They can be generic when directed at highly conserved sequences that cross different classes of organisms or they can be highly specific. In a BW context, gene probes are usually directed against a virulence factor or some signature sequence which distinguishes it from related non-pathogenic strains. While gene probes can be used to detect the presence of and indicate the identity of conventional biological agents (eg. viruses and bacteria), they can also be used to identify genes that have been transferred naturally or by human design into foreign vehicles (eg. cloning of BW toxin genes into innocuous organisms or encapsulation of infectious viral nucleic acid within man-made vesicles). While gene probes cannot be used to detect toxins directly, it is possible to detect signature genes or fragments thereof, if present in a sample, for example, a crude toxin preparation.

Gene probe identification of very small quantities of target material is made possible by using molecular biological techniques which increase the number of copies of a target sequence (amplification). One of these techniques, called polymerase chain reaction or PCR, uses short DNA sequences called primers that hybridize to the target nucleic acid and set the boundaries of the region to be amplified. The PCR process generates multiple copies of this region. If the primers complement unique or agent-specific sites on the target sequence, size analysis of the amplified fragment or amplicon, can be used as a means of identification. Confirmation that the correct DNA fragment has been amplified, can be obtained by probing the amplicon with a gene probe that is designed to bind specifically to the amplicon.

Initial studies at DRES have focussed on developing PCR primers and gene probes for Newcastle disease virus. This has been done for two reasons. First, NDV has been developed as a BW viral simulant for use in field experiments at DRES [1]. Second, many viruses that could be potential BW threats are RNA viruses. Since the genomic nucleic acid component of NDV is RNA, NDV serves as a useful model for developing methodologies for RNA virus identification.

PCR primer and gene probe design has been aimed at the major nucleocapsid protein (NP) gene of NDV because it has been suggested that the nucleocapsid structure unit is a necessary requirement in the template activity of negative-stranded RNA viruses [2]. As such, the nucleocapsid protein gene, being a major component of the nucleocapsid structure, should be a good target with which to design gene probes against negative-stranded RNA viruses.

We generated a 673 base pair DNA fragment of the NP gene from NDV strain B1 genomic RNA using reverse transcription PCR (RT-PCR) for other DRES studies [3,4]. Reverse transcription is required to convert the genomic RNA to DNA prior to PCR amplification as the enzyme used in the PCR process is a DNA polymerase which requires a DNA template for activity. This report describes the process of preparing this 673 bp gene sequence and cloning it into a viral DNA vector molecule to construct a recombinant viral vector DNA molecule. The recombinant DNA is easily stored within and recovered from a bacterial host cell line, thereby allowing us the capability to maintain and recover the cloned NDV sequence in unlimited quantities for future studies.

MATERIALS AND METHODS

Reagents

Common reagents used in this study included 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), isopropanol and nitro blue tetrazolium (NBT) from Fisher Scientific (Edmonton, AB). ATP, bromphenol blue, chloroform, dithiothreitol, ethidium bromide, ethylene diamine tetraacetic acid (EDTA), ethyleneglycol-bis [β -aminoethyl ether] tetraacetic acid (EGTA), glucose, glycerol, isopropylthio- β -d-galactoside (IPTG), isoamyl alcohol, lysozyme, magnesium chloride, mineral oil (heavy), phenol, polyethylene glycol, RNase A, sodium acetate, sodium chloride, sodium citrate, sodium dodecyl sulfate

(SDS), sodium hydroxide, sodium N-lauroylsarcosine, sucrose, Tris, Triton X-100 and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) were obtained from Sigma Chemical Co. (St. Louis, MO). N-butanol was obtained from Caledon Laboratories Ltd. (Georgetown, ON). Ethanol was purchased from Kodak International Biotechnologies Inc. (New Haven, CT).

Competent *E. coli* DH5 α F' cells, used for transformation of recombinant DNA, restriction enzymes *EcoR* I and *Pst* I and their commercially supplied reaction buffers, Low Melting Point (LMP) ultrapure agarose and ultrapure agarose were obtained from Gibco-BRL Life Technologies (Burlington, ON).

DNA molecular weight markers (λ phage DNA digested with *Hind* III restriction enzyme) and T4 DNA Ligase were purchased from Pharmacia-LKB (Baie d'Urfe, Quebec).

Calf intestinal alkaline phosphatase (CIP), lysozyme, *SnaB* I restriction enzyme and a commercially supplied buffer (buffer M), M13mp18 RF cloning vector DNA and reagents for southern blotting (nylon membranes, BMC blocking reagent and α -fluorescein-alkaline phosphatase Fab conjugate (α -F-AP)) were purchased from Boehringer Mannheim Company (Laval, Quebec).

PCR and RT-PCR reaction components (other than AMV reverse transcriptase, RNasin and primers) were obtained from Bio/Can Scientific (Mississauga, ON). AMV reverse transcriptase was obtained from Life Sciences (St. Petersburg, Florida). RNasin was obtained from Fisher-Promega (Edmonton, AB). PCR primers and oligonucleotide gene probes were synthesized by the Regional DNA Synthesis Laboratory of the University of Calgary (Calgary, AB). OligoTM Primer Analysis Software program version 4.1 from National Biosciences (Plymouth, MN) was used for primer and gene probe design based on sequence information for NDV strain D26 [5]. Figure 1 describes their sequences and locations. Restriction enzyme site selection and sequence analysis was performed using PC Gene version 6.5 software from Intelligenetics Incorporated (Mountainview, CA).

Bacterial growth media (broth, agar plates, top agar) was purchased from Difco (Detroit, MI).

Virus stocks of NDV strain B1 were obtained from the DRES collection (Ralston, AB).

All water used in this study was either triple distilled, deionized water which was made sterile by autoclaving or nuclease-free water from Promega-Fisher Scientific (Edmonton, AB).

Buffers

Assay buffer 1:

2x SSC, 0.1% SDS (pH 7.8)

Assay buffer 2:

0.1x SSC, 0.1% SDS (pH 8.0)

Assay buffer 3:

100 mM Tris, 150 mM NaCl (pH 7.5)

Assay buffer 4:

100 mM Tris, 150 mM NaCl, 0.5% BMC blocking reagentTM (pH 7.5)

Assay buffer 5:

100 mM Tris, 150 mM NaCl, 0.5% BMC blocking reagentTM (pH 7.5),
anti-fluorescein-alkaline phosphatase Fab conjugate at a concentration
of 300 mU/mL (1/2500 dilution of commercial stock)

Assay buffer 6:

100 mM Tris, 100 mM NaCl, 50 mM MgCl₂ (pH 9.5)

Assay buffer 7:

10 mM Tris, 1 mM EDTA (pH 8.0).

1x BRL restriction enzyme buffer 2:

50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl

1x BRL restriction enzyme buffer 3:

50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM NaCl

Color development solution:

100 mM Tris, 100 mM NaCl, 50 mM MgCl_2 (pH 9.5), 0.34 mg/mL NBT
and 0.17 mg/mL BCIP

10x gel loading buffer:

0.1% bromophenol blue, 1.0% SDS, 0.1 M EDTA pH 8.0, 50% glycerol

Hybridization buffer:

5x SSC, 0.1% Na-salt N-lauroylsarcosine, 0.02% SDS, 1% BMC blocking
reagentTM

LB broth:

10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl

LB plate:

1.5% agar, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl

LB top agar:

0.7% agar, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl

Ligation buffer:

50 mM Tris (pH 7.6), 10 mM MgCl_2 , 5% polyethylene glycol,
1 mM ATP, 1 mM dithiothreitol

Lysis solution:

0.2 M NaOH, 0.5% SDS

Lysis buffer:

50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA and 2 mg/mL
lysozyme

1 x PCR buffer (nested PCR)

50 mM KCl, 10 mM Tris (pH 9.0), 2 mM MgCl_2 , 0.1% Triton X-100,
200 μM of each of dATP;dTTP;dCTP;dGTP, 0.5 μM NDVNP-PR7
primer, 0.5 μM NDVNP-PR8 primer, 0.025 units/ μL Taq Polymerase

1x RT-PCR buffer

50 mM KCl, 10mM Tris (pH 9.0), 2mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTP mixture of dATP:dTTP:dGTP:dCTP, 0.2 µM NDVNP-PR5, 0.2 µM NDVNP-PR6, 0.2 µM Rnasin, 0.025 U/µL AMV reverse transcriptase and 0.025 U/µL Taq polymerase.

1x *Sna*B I restriction enzyme buffer (Buffer M):

10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 1 mM dithioerythritol

1x SSC:

0.15 M sodium chloride, 0.15 M sodium citrate buffer (pH 7.0)

STET buffer :

8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris (pH 8.0)

TAE electrophoresis buffer:

40 mM Tris, 20 mM sodium acetate, 1 mM EDTA (pH 7.2)

TE buffer:

10 mM Tris, 1 mM EDTA (pH 7.5)

Methods *Preparation of Insert DNA for Cloning*

The gene fragment that was cloned into the M13mp18 RF viral vector in this study was a double-stranded DNA fragment of 673 base pairs, prepared by RT-PCR amplification of purified genomic RNA from NDV strain B1 as follows.

NDV B1 RNA was purified according to procedures outlined elsewhere [3]. Forty µL of stock RNA (2.7×10^{10} molecules) was added to 360 µL of 1.1x RT-PCR buffer master mix to give a final 1x RT-PCR buffer concentration. Four 90 µL volumes were then aliquotted into separate oil-less PCR tubes (Bio/Can, Mississauga, Ontario). Three negative controls (30 µL each) were run in parallel, in which 3 µL of water were added to 27 µL of 1.1x RT-PCR buffer master mix (template-free). Prior to placing the PCR tubes into a programmable

DNA thermal cycler (Perkin Elmer Cetus), the cycler was allowed to warm up for 30 min and the diagnostic "auto-tune" program was performed. A drop of mineral oil was added per well and the thermal cycler was pre-warmed to 42°C. The PCR tubes were placed into the wells and incubated at 42°C for 1 h for the reverse transcription reaction. The following PCR thermal cycling reaction was then initiated: 94°C for 5 min; 94°C for 1 min + 47°C for 2 min + 72°C for 3 min (30 cycles); 72°C for 7 min; 4°C hold.

Samples and controls were analyzed by horizontal agarose gel electrophoresis against molecular weight standards (λ -Hind III DNA) for the presence or absence of the 673 bp amplified product. A 2 μ L aliquot from each tube was mixed with a 2 μ L of 10x gel loading buffer and brought to a total volume of 20 μ L with water. Ten μ L of this mixture was loaded onto a 1% agarose gel (0.75 g of agarose in 75 mL of TAE electrophoresis buffer containing 0.5 μ g/mL ethidium bromide). The samples were subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 1-2 h at room temperature in TAE electrophoresis buffer without ethidium bromide. The gel was visualized by UV illumination on a FotodyneTM UV light box (Bio/Can Scientific, Mississauga, ON). The sample tubes containing amplified product were combined, mixed with a 1/10 volume of 10x gel loading buffer and subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 2-3 h at 4°C through a 2% low melting point agarose gel. The gel was briefly exposed to UV light (prep setting) to visualize the amplified 673 bp band for excision. The band was excised from the gel and placed into 1.5 mL microcentrifuge tubes. A 1/3 volume of TE buffer was added, based on an estimation of the volume of the excised gel material using a specific density of 1.0 g/mL. The tubes were placed in a 65°C waterbath for 15 min to melt the agarose. The tubes were cooled to room temperature for 2-5 min. A 1/3 volume of water-saturated phenol was added, the tube was vortexed for 30 sec and centrifuged at 16,000 x g for 5 min. The top aqueous layer was transferred to new tubes and subjected to a second extraction with water-saturated phenol. The aqueous layer was removed and then extracted several times with an equal volume of dry n-butanol (vortexed for 30 sec and centrifuged for 1 min at 16,000 x g for each extraction). The extractions were repeated until the total volume of the aqueous layer was 1/10 the starting volume. The DNA was precipitated from the aqueous layer by adding a 1/10 volume of 3M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol, followed

by incubation at -20°C for 1 h or at -70°C for 30 min. The precipitate was pelleted by centrifugation at 16,000 x g for 30 min. The pellet was washed once with 70% ethanol, pelleted again by centrifugation at 16,000 x g for 30 min, vacuum-dried (10-30 min) and finally re-suspended in TE buffer or nuclease-free water. The DNA was analyzed for quality and quantity by running a sample on a regular 1% agarose gel at room temperature. The concentration of sample DNA was obtained by comparing band intensities to molecular weight markers of known concentration (λ DNA digested with Hind III).

Restriction Enzyme Digestion of Insert and Vector DNA

M13mp18 RF cloning vector DNA and purified 673 bp NP gene fragment DNA were treated separately with *EcoR* I and *Pst* I restriction enzymes as follows. Two hundred to five hundred ng of DNA were treated for 1h at 37°C with 10 units of *Pst* I restriction enzyme in 1x BRL restriction enzyme buffer 2 in a total volume of 30 μ L. Five μ L of 0.5 M NaCl, 1 μ L of 10 units/ μ L *EcoR* I restriction enzyme and 4 μ L of nuclease-free H₂O were then added to the reaction mixture and incubated for 1 h at 37°C. The reaction was quenched by adding a 1/10 volume of 10x gel loading buffer. Each sample (M13mp18 RF and NDVNP673 DNA) was loaded onto separate low melting point agarose gels. The DNA was separated by gel electrophoresis, visualized by UV illumination to locate the position of the DNA fragments of interest on the gel, and then recovered from the gel using the melting method already described. The DNA was then analyzed for quality and quantity by agarose gel electrophoresis through a 1% (wt/vol) agarose gel. Quantitation of sample DNA was performed by comparing band intensities to commercial molecular weight markers of known concentration.

EcoR I/*Pst* I treated M13mp18 RF DNA was further treated with calf intestinal alkaline phosphatase (CIP) to dephosphorylate the 5' end, thereby reducing self-ligation and concatamerization of the vector DNA. Dephosphorylation was carried out by adding 1 unit of CIP per μ g of vector DNA in 50 mM Tris (pH 8.0), 0.1 mM EDTA. The reaction was carried out at 37°C for 15 min and then at 56°C for 15 min. Another unit of CIP was added per μ g and the incubation repeated. The reaction was quenched by adding EGTA to a final concentration of 10 mM and heating the mixture at 65°C for 45 min.

Ligation of Insert and Vector DNA

The ligation reaction was performed using 12 fmoles of *EcoR* I/*Pst* I treated M13mp18 RF DNA (57 ng) and 4 fmoles of *EcoR* I/*Pst* I treated NDVNP673 DNA (1.85 ng) to give a molar ratio of 3:1 vector to insert, respectively. The reaction was carried out in ligation buffer with 0.2 units of T4 DNA ligase at 14°C for 16 h in a total volume of 12 µL. The ligation reaction mixture was diluted 5-fold with H₂O. Ten µL of the ligation mixture was added to 90 µL of competent *E. coli* DH5αF' cells and incubated on ice for 30 min. The cells were heat-shocked at 42°C for 2 min and then incubated on ice for 5 min. Serial 10-fold dilutions up to 10⁻⁵ were made to a total volume of 100 µL for each dilution. Each dilution (100 µL) was added to 3 mL of LB top agar containing 0.4 mM IPTG, 400 µg/mL X-gal and 100 µL of uninfected DH5αF' lawn cells. This was poured onto an LB plate containing about 20 mL of solidified agar, giving an approximate final concentration of 0.05 mM IPTG and 50 µg/mL X-gal per plate. The plates were incubated at 37°C overnight. Clear plaques, in the presence of IPTG and X-gal, were indicative of clones containing the recombinant DNA.

Small Scale Purification of Recombinant DNA

Individually selected recombinant plaques were transferred to 5 mL of LB broth along with a loop of uninfected *E. coli* DH5αF' cells. This was incubated overnight at 37°C and 200 rpm in a New Brunswick shaker incubator. A 1 mL aliquot was transferred into a 1.5 mL microcentrifuge tube and centrifuged for 5 min to pellet the cells. The supernatant was poured off and the cells were resuspended in 50 µL of STET buffer. Two µL of a 10 µg/µL lysozyme solution was added, mixed briefly, and incubated at room temperature for 5 min. The tube was placed in a boiling water bath for 1 min and immediately centrifuged at 16,000 x g for 10 min at room temperature. The supernatant was precipitated by adding an equal volume of isopropanol, mixing and leaving at -20°C for 1 h or -70°C for 30 min. The DNA was pelleted by centrifugation at 16,000 x g for 30 min and then resuspended in 20 µL of TE buffer and stored at -20°C.

Large Scale Purification of Recombinant DNA

Individually selected recombinant plaques were transferred along with 2 mL of an overnight culture of *E. coli* DH5 α F' cells, into 200 mL of LB broth and grown for 6 hours at 37°C with shaking in a New Brunswick shaker at 200 rpm. Four 40 mL aliquots of liquid culture were placed in Oakridge tubes (Nalgene-Fisher, Edmonton, Alberta) and cooled on ice for 15 min. The cells were centrifuged at 4000 x g for 10 min (4°C), in a Beckman high-speed centrifuge. The cellular pellet was washed with TE buffer and then centrifuged again. Each pellet was resuspended in 4 mL of lysis buffer, mixed and incubated on ice for 30 min. Then, 8.5 mL of lysis solution was added, mixed gently by inversion until a visible clearing was observed and then placed on ice for 30 min. Then 5.3 mL of 3 M sodium acetate (pH 5.0) was added, mixed gently and placed on ice for 1 h. The final suspension was centrifuged at 10,800 x g for 20 min at 4°C to pellet the precipitated chromosomal material which was discarded. The supernatant was transferred to a clean tube and centrifuged as above until no chromosomal material remained in the supernatant. Heat-treated RNase A (15 min at 100°C) was added to the supernatant to a concentration of 100 μ g/mL and incubated at 37°C overnight. One volume of water-saturated phenol was added, mixed for 1 min and then centrifuged at 4000 x g for 5 min. The top aqueous layer was removed and extracted with 1 volume of a chloroform:isoamyl alcohol solution (24:1) by mixing for 1 min, then spinning in a centrifuge at 4000 x g for 5 min. The top aqueous layer was transferred to a clean tube and a 1/10 volume of 3 M sodium acetate (pH 5.0) was added, followed by 2 volumes of 95% ethanol. This was mixed and stored for a minimum of 1 h at -20°C. The precipitated material was pelleted by centrifugation at 10,800 x g for 30 min. The pellet was washed with 70% ethanol, pelleted by centrifugation (10,800 x g for 30 min), vacuum dried (10 min) and resuspended in a total of about 500 μ L of TE buffer. Agarose gel electrophoresis was used to assess the purity and quantity of recombinant DNA.

EcoR I/Pst I Restriction Enzyme Digestion of Recombinant DNA

Double digestions were performed in one step in 1x BRL Buffer 3. Final concentrations were typically 10-50 ng/ μ L DNA, 1 unit/ μ L *Pst* I and 1 unit/ μ L *EcoR* I. Digestions were carried out for 1-2 hours at 37°C.

SnaB I Restriction Enzyme Digestion

SnaB I restriction enzyme digestion was used to linearize M13mp18 RF and recombinant DNA for electrophoresis and gene probe studies. Digestions were carried out in 1x buffer M. Final concentrations were typically 10-50 ng/ μ L DNA and 0.05 to 0.5 units/ μ L of *SnaB I*. Digestions were carried out for 1-2 hours at 37°C.

Southern Blot Analysis

The gene probe used for southern blot analysis of the recombinant DNA was NDVNP-PB4, a 5'-fluoresceinated, 20 base oligonucleotide probe (Figure 1). This sequence was derived from a highly conserved region within the NP gene of NDV (Figure 2). Alignment of this region among viruses in the Paramyxoviridae family indicated 100% homology among the two NDV strains but only 20-25% homology with the other sequences.

Samples to be analyzed by southern blotting with the NDVNP-PB4 probe were first subjected to electrophoresis on 1% agarose gels to separate the DNA fragments. After run times of 1.5 to 2 h, the DNA was denatured to single strands by soaking the gels in 0.5 M NaOH (3 changes for 10 min each) with shaking. The gels were then transferred to 0.5 M Tris-HCl pH 7.0 (3 changes for 10 min each) with shaking, followed by soaking in 2x SSC for 30 min. DNA from the gel was transferred to a nylon membrane using the gel transfer setup similar to that described elsewhere [6]. The gel transfer materials (wick, nylon membrane and filters) were presoaked in 2x SSC. The transfer was allowed to go overnight (16-24 h) using 10x SSC buffer. The membrane was removed, air-dried for 30 min and then exposed to UV light for 5 min on a UV light box (FotodyneTM), using the preparative setting, to cross-link the DNA to the membrane.

The membranes were placed into hybridization bags containing 0.2 mL/cm² hybridization buffer (without probe) and incubated in a water bath shaker at the hybridization temperature of interest for 1 h to block un-reacted DNA binding sites on the membrane. The buffer was removed and replaced with 0.05 mL/cm² of fresh hybridization buffer containing NDVNP-PB4 probe DNA (probe material was always denatured by heating at 100°C for 10 min and chilling on ice for 5 min beforehand). The membranes were incubated at the required temperature for 1 hr in a shaking water bath. The membranes were then washed 2x for 5 min in 0.5

mL/cm² of assay buffer 1 at room temperature to remove unbound probe. This was followed by two 5-15 min washes in 0.5 mL/cm² of assay buffer 2 at temperatures at or above room temperature. The remaining washes were done at room temperature. Membranes were prepared for detection by first rinsing briefly (1 min) in assay buffer 3. This was followed by washing in 1 mL/cm² of assay buffer 4 for 30 min and then 1 mL/cm² of assay buffer 5 for 30 min. This was followed by two washes in 1 mL/cm² of assay buffer 3 for 15 min each and then equilibration in 1 mL/cm² of assay buffer 6 for 5 min. The membranes were incubated in the dark with 0.1 mL/cm² of color development solution. Color development was monitored and allowed to proceed anywhere from 1 to 24 hours at which time the reaction was quenched by removing the membrane from the color development solution and washing the membrane in 1 mL/cm² of assay buffer 7.

Nested PCR Amplification

Nested PCR amplification reactions were carried out in 1x PCR buffer using *SnaB* I linearized recombinant DNA as the template. Temperature cycling was performed on 20 or 100 µL volumes of PCR reaction aliquots in 1.5 mL oil-less tubes (Bio/Can Scientific, Mississauga, ON). The thermal cycling program for the reaction was 94°C for 5 min, followed by 30 cycles of 94°C for 1 min + 55°C for 1 min + 72°C for 1 min. The reaction was completed by heating to 72°C for 7 min and then cooling to 4°C.

RESULTS AND DISCUSSION

Cloning

A schematic diagram of the cloning strategy used in this study is presented in Figure 3.

The first step in the cloning strategy was to generate the NDVNP673 DNA fragment by RT-PCR of purified genomic RNA from NDV strain B1 (Figure 4). Three of four replicate samples showed the presence of amplified material close to the theoretical amplicon size of 673 bp (about 660 bp). This material was considered to be the intended amplified product. There was no

evidence of amplification product for one of the four replicate samples indicating that this amplification reaction had failed¹. The negative control reaction, in which template RNA was not added, did not generate any visible DNA bands indicating that the RT-PCR reaction was not corrupted with previously amplified material.

The second step of the cloning process involved *EcoR* I/*Pst* I restriction digestion of the 673 bp amplified DNA (insert) and M13mp18 RF DNA (vector), ligation of the insert and vector reaction mixtures, and transformation of *E. coli* cells with the ligation reaction mixture. Clear plaques were evident for cells transformed with the ligation reaction mixture while cells transformed with control vector DNA, generated blue plaques. Clear plaques were indicative of clones containing the recombinant DNA due to the fact that the recombinant molecules lacked an intact *lac Z* gene coding region as a consequence of inserting the 673 bp NDV sequence within this region. The disrupted *lac Z* coding sequence failed to code for a functional gene product and thus the cells were unable to hydrolyze the colorless substrate X-gal into blue-colored product, thereby resulting in clear plaques. Conversely, cells transformed with the vector DNA which contained an intact *lac Z* sequence, were able to hydrolyze X-gal, resulting in blue-colored plaques.

*Restriction
Enzyme
Analysis of
Recombinant
DNA*

To confirm the presence of the 673 bp insert in the recombinant clones, DNA was isolated from six clear plaques using the small scale method and analyzed for the presence of the cloned insert by agarose gel electrophoresis following *EcoR* I/*Pst* I digestion (data not shown). There was considerable RNA present in the sample preparations due to the fact that no RNase digestion step was included in the method. The RNA overlapped at the position of the 673 bp fragment for most of the samples but fortunately, one of the six plaques was not obscured by the RNA band and a comparison of the untreated control with the *EcoR* I/*Pst* I treated sample suggested that this plaque contained the cloned insert. Consequently, this plaque was selected for large scale purification of recombinant DNA. Subsequent restriction enzyme analysis of the purified recombinant DNA indicated the presence of the 673 bp fragment in the recombinant clone (Figure 5, lane 9).

¹ We have experienced amplification failures in the past. A study of this phenomenon was performed [7] which suggested that amplification failures are likely a result of insufficient heat transfer between the temperature block of the cylinder and the reaction tube.

***Southern Blot
Analysis of
Recombinant
DNA***

Southern blot analysis of purified recombinant DNA (linearized with *SnaB* I) was performed using NDVNP-PB4 (Figure 6). There was no signal for the negative control vector DNA (Figure 6b, lanes 1-2) but there was a signal for the recombinant DNA (Figure 6b, lane 3 and 4) indicating that the cloned NDVNP673 insert was present in the recombinant vector DNA construct.

***Nested PCR
Amplification of
Recombinant
DNA***

Nested PCR amplification of *SnaB* I linearized recombinant DNA was carried out using NDVNP-PR7/8 primers to generate a 391 bp amplicon. Agarose gel analysis indicated the presence of a fragment of approximately 390 bases (Figure 7a), based on size estimation relative to molecular weight markers. This provided more evidence that the cloned fragment was the NDVNP673 fragment since it would have to contain the proper sequence to allow the nested amplicon to be generated. The NDVNP-PB4 gene probe was used to probe the 391 bp amplicon in a southern blot (Figure 7b). A positive signal was observed providing confirmation that the cloned NDVNP673 fragment was present in the recombinant molecule.

CONCLUSION

A 673 bp DNA fragment of the nucleocapsid protein gene of NDV B1 was shown to be successfully cloned into viral M13mp18 vector DNA to generate the recombinant M13mp18-NDVNP673 molecule based on clonal selection on indicator plates, restriction enzyme analysis, gene probe hybridization with an internal probe and PCR amplification using nested PCR primers.

Cloning this DNA sequence into M13mp18 RF DNA and maintaining it within the *E. coli* bacterial host, will allow us the capability to isolate large quantities of this sequence for future studies. In addition, the NP gene sequence was converted from RNA to DNA for cloning purposes and as a result, will be easier to work with, since ribonucleases are much more difficult to inhibit than deoxyribonucleases.

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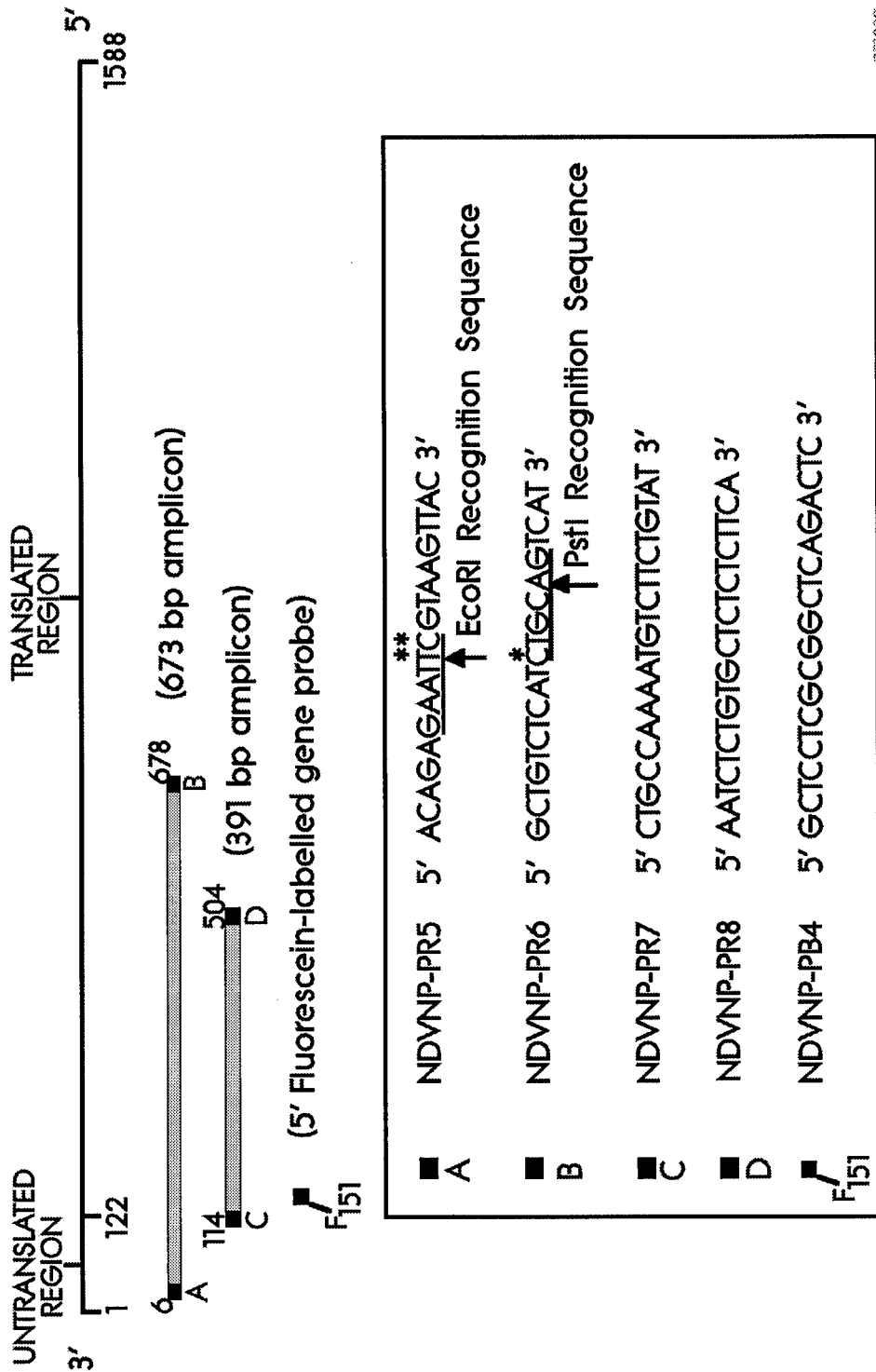


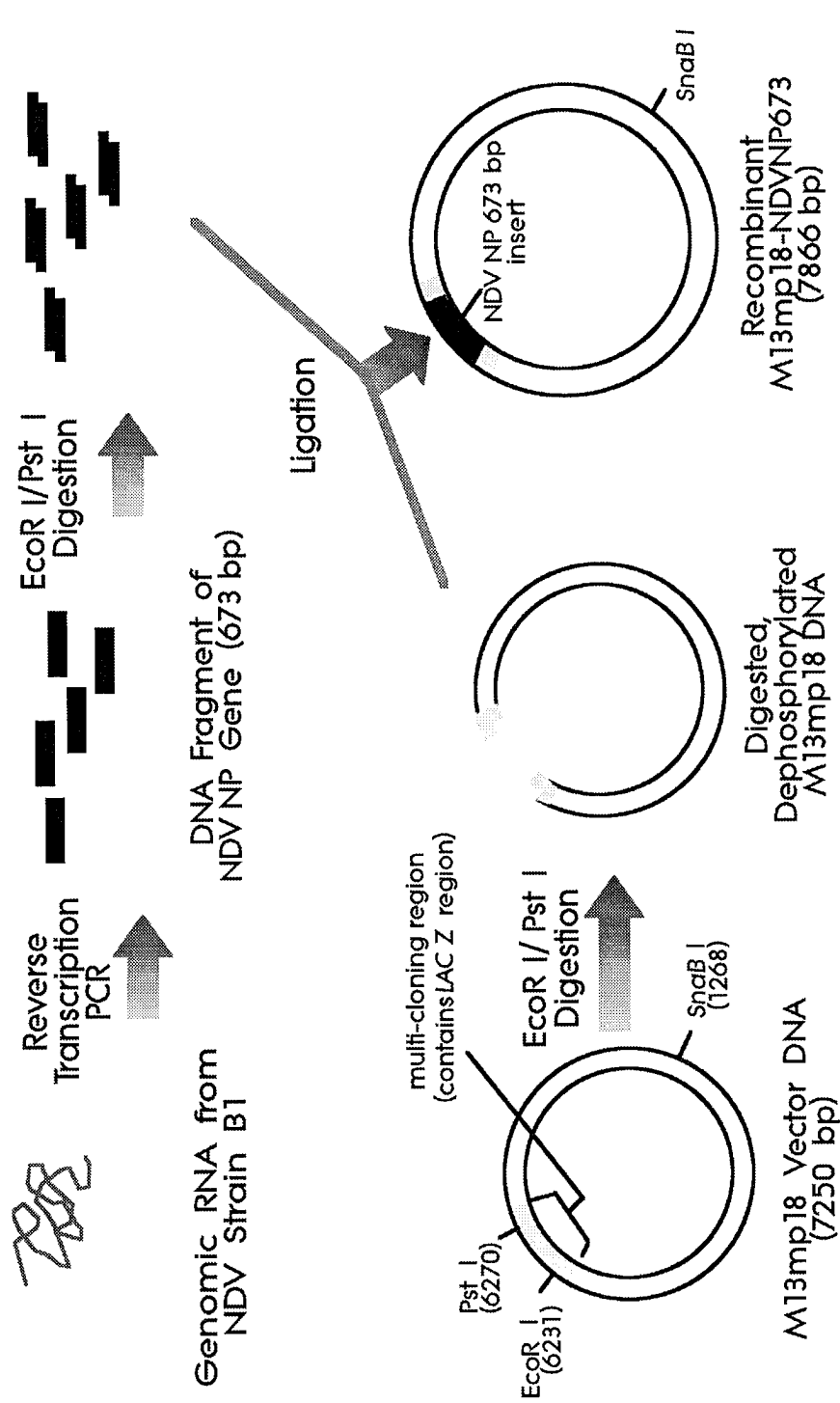
Figure 1

Schematic diagram of PCR primer sets and gene probe locations on the NDV NP gene (adapted from [5]). Asterisks (*) indicate base changes designed to introduce unique restriction enzyme recognition sites (underlined) within the amplified 673 bp DNA amplicon following PCR amplification.

	Sequence							% homology to NDV
Genus: Paramyxovirus								
NDV (D26)	GCT	CCT	CGC	GGC	TCA	GAC	TC	-
NDV (Beaudette C)	GCT	CCT	CGC	GGC	TCA	GAC	TC	-
Sendai	<u>ATT</u>	TAG	<u>CTC</u>	TAG	GAG	<u>GAG</u>	CG	25
Parinfluenza type 2	<u>ATT</u>	TAC	TAT	ACA	<u>ACA</u>	<u>GGA</u>	<u>GC</u>	25
Parinfluenza type 3	<u>ACG</u>	TAG	GCA	<u>AGA</u>	<u>AAA</u>	<u>CAT</u>	AA	20
Parinfluenza type 4a	<u>GTT</u>	<u>TCT</u>	TCA	<u>AAC</u>	AAC	TGA	AG	25
Parinfluenza type 4b	<u>ATT</u>	<u>TCT</u>	TCA	<u>AAC</u>	AAC	TGA	AG	20
Genus: Morbillivirus								
Measles	<u>GTT</u>	<u>CAA</u>	AAG	AAA	<u>CAA</u>	<u>GGA</u>	CA	25
Genus: Pneumovirus								
Respiratory syncytial virus	TAC	<u>ACT</u>	<u>CAA</u>	CAA	<u>AGA</u>	TCA	<u>AC</u>	25

Figure 2

Alignment of partial NP gene sequences representing eight different species and three different genera within the Paramyxoviridae family: NDV D26 [5]; NDV Beaudette C [8]; Sendai virus [9]; Parainfluenza type 2 [10]; Parainfluenza type 3 [11]; Parainfluenza type 4a and 4b [12]; Measles virus [13] and Respiratory syncytial virus [14]. Conserved nucleotides relative to the NDV sequences are underlined.



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Figure 3

Schematic diagram of the cloning strategy used in the construction of recombinant M13mp18-NDVNP673 DNA.

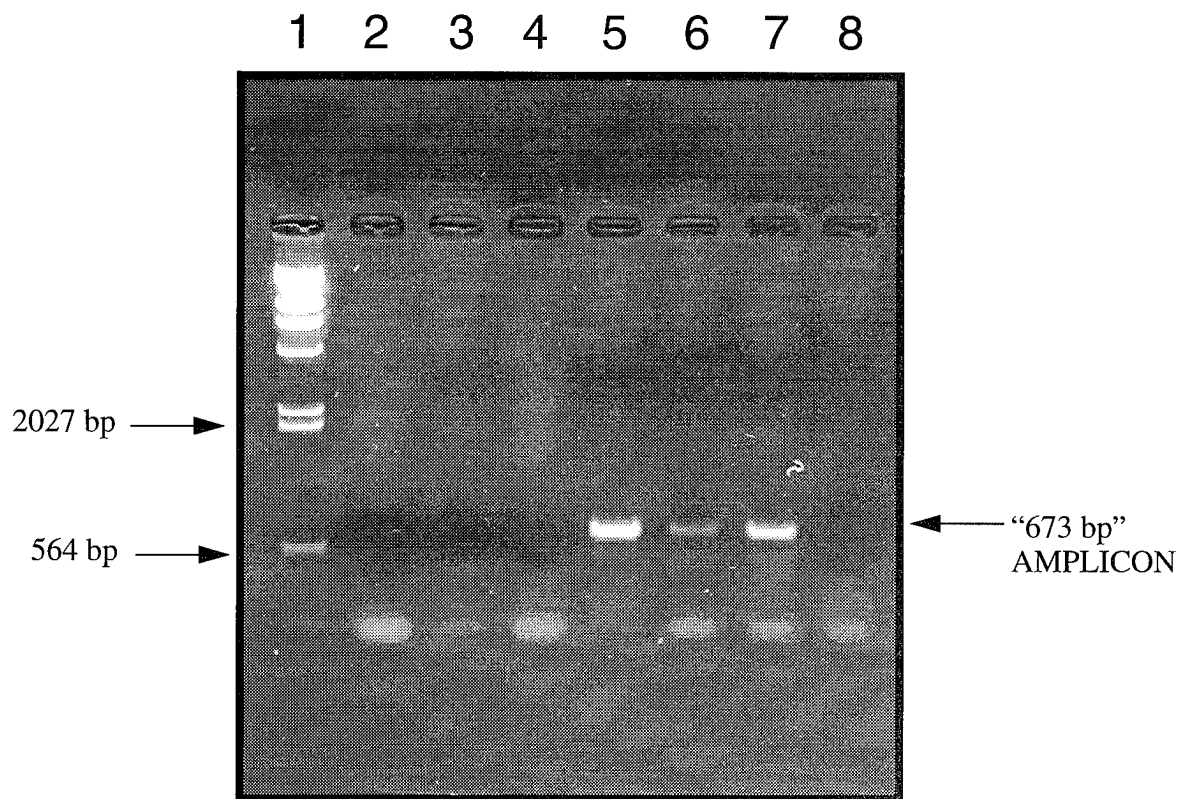


Figure 4

Horizontal agarose gel electrophoresis of NDVNP673 DNA following reverse transcription PCR of NDV B1 RNA. Conditions: 1% agarose gel run at 60V for 2h in TAE electrophoresis buffer. **Lane 1:** λ -Hind III molecular weight markers; **lanes 2-4:** negative control (no template); **lanes 5-8:** positive control (NDV B1 RNA).

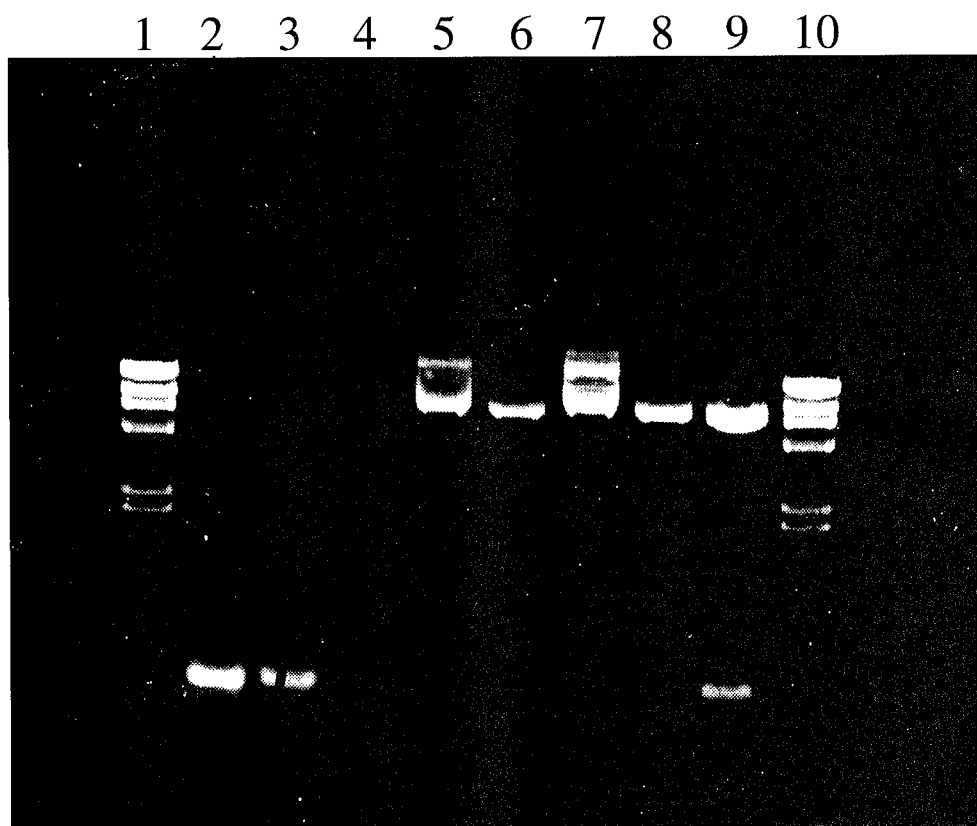


Figure 5

EcoR I/*Pst* I restriction enzyme digestion analysis of recombinant DNA. Conditions: 1% agarose gel, 60V for 2h in TAE electrophoresis buffer. **Lanes 1 and 10:** λ -Hind III molecular weight markers (1 μ g). **Lanes 2-4:** 500 ng, 50 ng and 5 ng of purified 673 bp DNA, respectively. **Lane 5:** untreated vector DNA. **Lane 6:** *SnaB* I linearized vector DNA. **Lane 7:** untreated recombinant DNA. **Lane 8:** *SnaB* I linearized recombinant DNA. **Lane 9:** *EcoR* I/*Pst* I treated recombinant DNA.

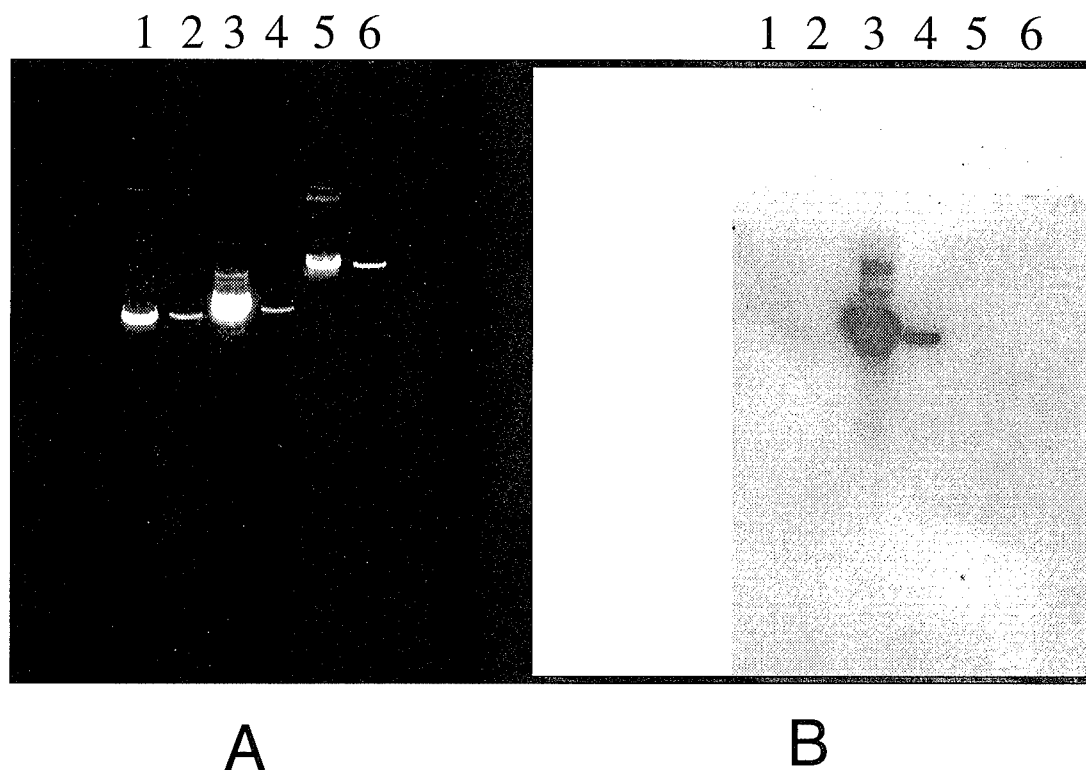


Figure 6

Southern blot analysis of recombinant DNA and vector DNA with an NDV oligonucleotide gene probe. **Lanes 1 and 2:** *SnaB* I linearized vector DNA (100 ng and 10 ng respectively). **Lanes 3 and 4:** *SnaB* I linearized recombinant DNA (500 ng and 10 ng respectively). **Lanes 5 and 6:** λ DNA (500 ng and 10 ng respectively). (A) Agarose gel electrophoresis. (B) Southern blot analysis under low stringency (T_m -30°C): 40 ng/mL NDVNP-PB4 oligonucleotide probe hybridized at 54°C for 1h; 25°C post-hybridization wash in 0.1x SSC/0.1% SDS; 14 h color development.

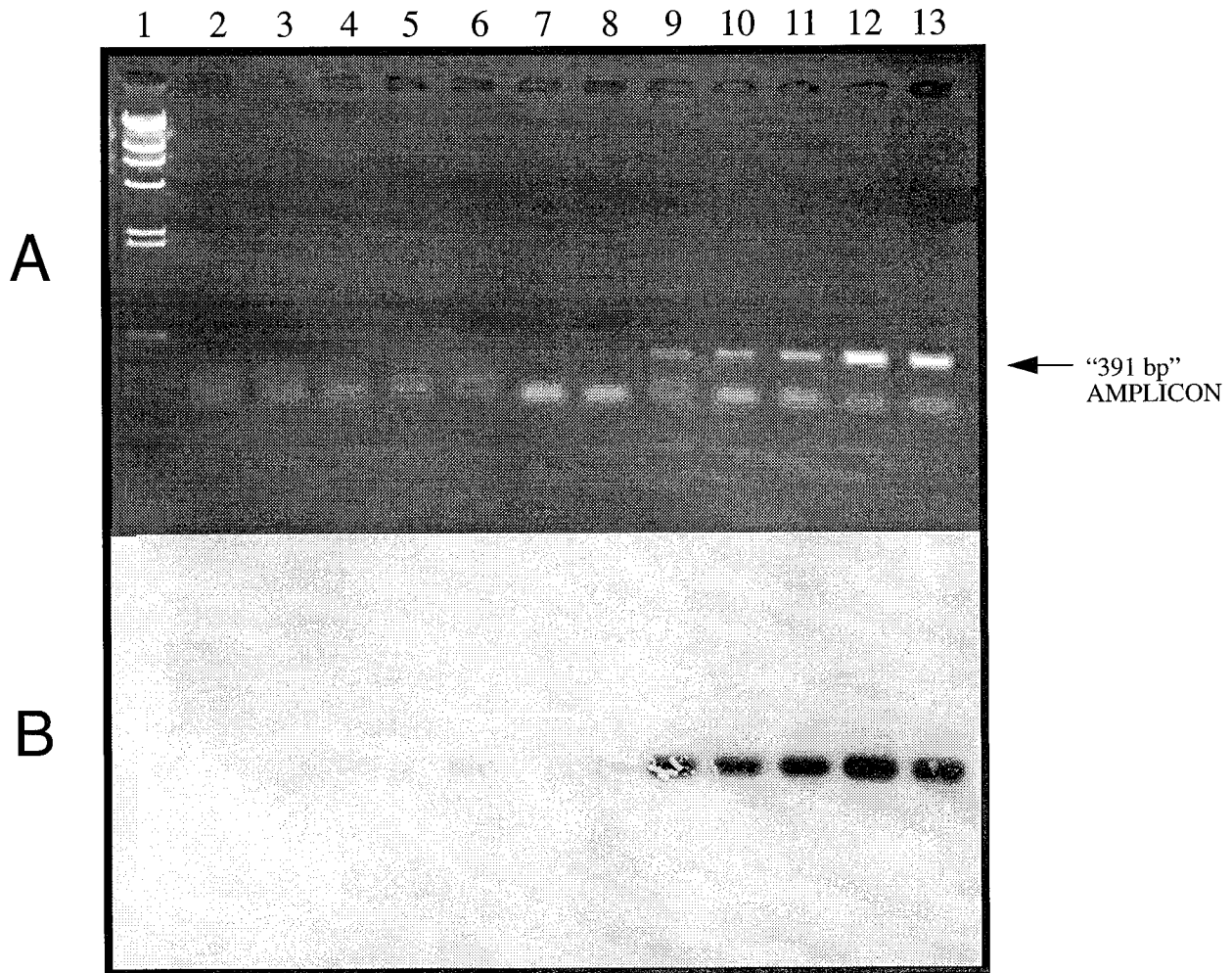


Figure 7

Nested PCR reactions of recombinant DNA. **Lane 1:** λ -Hind III molecular weight markers (1 μ g). **Lane 2-13:** recombinant DNA template starting at 4×10^{-4} molecules to 4×10^6 molecules in duplicate 100-fold increments. (A) Agarose gel electrophoresis. (B) Southern blot analysis under highly stringent conditions ($T_m - 10^\circ\text{C}$): 94 ng/mL of NDVNP-PB4 oligonucleotide probe hybridized at 58°C for 1 h; 48°C post hybridization wash in 0.1x SSC/0.1% SDS; 16 h color development.

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This report describes the procedures used to clone a 673 base pair gene fragment of the major nucleocapsid protein gene of Newcastle disease virus into a viral vector molecule for the purpose of maintaining a stable, long-term, renewable source of this target sequence for gene probe studies.

The gene fragment was prepared by reverse-transcription polymerase chain reaction of Newcastle disease virus RNA and was cloned into a viral DNA vector M13mpl8 RF to produce a recombinant DNA molecule. The cloned fragment was shown to be present in the recombinant clones based on (i) clonal selection on indicator plates; (ii) restriction enzyme analysis; (iii) gene probe analysis and (iv) nested PCR amplification.

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